

# **Epidemiology of Bovine Viral Diarrhoea Virus and Bovine Herpesvirus type 1 Infections in Dairy Cattle Herds**

**Evidence of self-clearance and detection of infection  
with a new atypical pestivirus**

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## Abstract

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Bovine viral diarrhoea virus (BVDV) and bovine herpesvirus type 1 (BHV-1) infections are endemic and cause disease in cattle populations worldwide. The losses due to the diseases are high in affected countries and must be regarded as a serious animal welfare problem. In Sweden BHV-1 has been eradicated and more than 98% of the herds are BVDV free. This long-term study was conducted to obtain knowledge of relevance for control of both these infections in dairy herds in Thailand. The epidemiological pattern of BVDV and BHV-1 infections was studied in 186 dairy herds by repeated bulk tank milk (BTM) analysis. A subset of 11 herds was selected for individual testing. The serum and BTM were tested for antibodies to the viruses using indirect ELISA tests. The results demonstrated a self-clearance process of both infections at the herd/regional level, without any control measures. There was an apparently lack of reactivation of BHV-1 infected animals. A significant association of BHV-1 and BVDV indicate that common risk factors are associated with seropositivity. Factors discussed were the serological status of imported cows and artificial insemination activities.

A low incidence rate of BVDV was found among breeding cattle in 5 out of 11 herds. One herd, however, had a high seroconversion rate to BVDV and a viraemic calf was identified by an E<sup>ms</sup> capture ELISA. Genetic characterization showed that this calf was infected with a virus closely related to a recently described atypical pestivirus (HoBi) detected in a foetal calf serum from Brazil. Comparative neutralisation tests suggest that seroconversions in this herd were caused by the 'HoBi-like' virus and that it has been recently introduced to the region. Further studies must be performed to elucidate how this virus has been introduced to the cattle population in Thailand and it might have been spread to cattle all over the world.

However, this study shows that the self clearance process in Thai dairy herds of both BVDV and BHV-1 infections will probably continue if there is awareness of biosecurity. Imported animals and biological products should be secured free from both BVDV and BHV-1.

*Keywords:* BVDV; pestivirus, HoBi, BHV-1; herpesvirus, self-clearance; control

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## Papers I-V

The thesis is based on the following papers:

I. Kampa, J., Ståhl, K., Moreno-López, J., Chanlun, A., Aiumlamai, S. and Alenius, S. (2004). BVDV and BHV-1 infections in dairy herds in northern and northeastern Thailand. *Acta Veterinaria Scandinavica* (45), 181-192.

II. Kampa, J., Alenius, S., Emanuelson, U., Chanlun, A. and Aiumlamai, S. BHV-1 and BVDV infections in dairy herds: evidence of self-clearance and detection of seroconversion against a new atypical BVDV strain. (Manuscript)

III. Kampa, J., Ståhl, K., Renström, L.H.M. and Alenius, S. Evaluation of the suitability of a commercial E<sup>ms</sup>-capture ELISA for detection of BVDV in cattle serum samples within a control programme. (Submitted).

IV. Ståhl, K., Kampa, J., Baule, C., Isaksson, M., Moreno-López, J., Belák, S., Alenius, S. and Lindberg, A. (2005). Molecular epidemiology of bovine viral diarrhoea during the final phase of the Swedish BVD-eradication programme. *Preventive Veterinary Medicine* (72) 103-180.

V. Ståhl, K., Kampa, J., Alenius, S., Persson Wadman, A., Baule, C., Aiumlamai, S. and Belák, S. Natural infection of cattle with an atypical 'HoBi'-like pestivirus: Implications for BVDV control and for the safety of vaccines and other biological products. (Manuscript).

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## Abbreviations

AI	artificial insemination
BDV	border disease virus
BHV-1	bovine herpesvirus type 1
BTM	bulk tank milk
BVDV	bovine viral diarrhoea virus
CI	confidence interval
COD	corrected optical density
cp	cytopathic
CSFV	classical swine fever virus
DLD	Department of Livestock Development, Thailand
DNA	deoxyribonucleic acid
ELISA	enzyme-linked immunosorbent assay
kb	kilobase
IBP	infectious balanoposthitis
IBR	infectious bovine rhinotracheitis
IPV	infectious pustular vulvovaginitis
IPX	immunoperoxidase test
MAb	monoclonal antibody
MC	milk collection centre
ncp	non-cytopathic
NCR	non-coding region
NS protein	non-structural protein
nt	nucleotide
OD	optical density
OIE	<i>Office International des Epizooties</i> World Organisation for Animal Health
ORF	open reading frame
PCR	polymerase chain reaction
PI	persistently infected
PP	percent positivity
RNA	ribonucleic acid
RT-PCR	reverse transcription- polymerase chain reaction
SVA	National Veterinary Institute, Sweden
VN	virus neutralisation



# Introduction

## Dairy production in Thailand

The dairy industry in Thailand today was first established in the early 1960s in the central region of Thailand, through a royal co-operative project between the Thai and the Danish governments. During the following decades the dairy cattle population and the milk production increased but not sufficiently to meet the increasing market demand for milk and milk products. Therefore, since the early 1990s, the Royal Thai government has encouraged farmers to start small holder dairy farms in other parts of the country, including the north-eastern provinces. Initially dairy cattle were introduced from central Thailand but as the numbers were inadequate, pregnant cows and heifers were imported from several other countries, mainly Australia and New Zealand. In 2005, the total number of dairy cows in Thailand was 478 386, a 3.5-fold increase since 1989. The average size of a dairy herd in the country is 9 milking cows/herd and the mean daily milk production is 9.6 kg/cow (DLD, 2004).

Khon Kaen, Udorn Thani and Sakon Nakorn provinces are situated in north-east Thailand (map in Paper I). There are 14,027 dairy cattle in 1,100 herds in these provinces that sell their milk to 9 milk collection centres (MCs). In general, the main issues that concern dairy farmers are sub-optimal milk production, high somatic cell counts, reproductive failure and health problems. These problems are due to a combination of causes such as humid hot weather, nutritional insufficiency and infectious diseases.

## Infection with bovine viral diarrhoea virus and bovine herpesvirus type 1 in cattle

Bovine viral diarrhoea virus (BVDV) and bovine herpesvirus type-1 (BHV-1) infections are spread in cattle populations all over the world. Serological surveys have revealed a high prevalence of antibodies to both BVDV and BHV-1 in the cattle populations of many countries including Thailand (Virakul *et al.*, 1997; Houe, 1999; Straub, 2001; Moennig, Houe & Lindberg, 2005; Solis-Calderon, Segura-Correa & Segura-Correa, 2005).

Infections with BVDV and BHV-1 cause losses from reproductive failures and increase the severity of secondary infections by other pathogens (Gibbs & Rweyemamu, 1977; Potgieter *et al.*, 1984a; Duffell & Harkness, 1985; Fray, Paton & Alenius, 2000; Straub, 2001; Ackermann & Engels, 2006). Effective control of BVDV and BHV-1, therefore, not only reduce the economic losses in production but also can prevent the suffering from disease and thus improve animal welfare. Since Thailand has signed the Free Trade Agreement with intensive milk production countries such as Australia and New Zealand, Thai dairy farmers must in order to be competitive improve both the dairy production and animal welfare. To achieve these aims, control programmes for infectious diseases are needed. At present, brucellosis, paratuberculosis and bovine tuberculosis are in eradication

schemes (Bureau of Disease Control and Veterinary Services, 2006) while foot and mouth disease, haemorrhagic septicaemia and anthrax are controlled by vaccination. The cows in Thailand are usually artificially inseminated with frozen semen produced in the country by the government and by private artificial insemination (AI) stations. Imported semen from many other countries has also been used. BVDV and BHV-1 are controlled as suggested by the OIE (2005) in all government AI stations, but only in a few number of private stations. After January 2006, AI stations that do not follow the regulation should be regarded as illegal semen producers (Bureau of Biotechnology in Livestock Production, 2005).

Before the introduction of control measures for BVDV and BHV-1 infections it is desirable to have knowledge of the epidemiology and impact of the infections in the region. This work in this thesis was conducted with the purpose to gain such knowledge about the epidemiology of BVDV and BHV-1 infections in small dairy herds in Thailand, as a background to future control efforts.

## **Bovine viral diarrhoea virus**

### *The virus*

Bovine viral diarrhoea virus is a heterogeneous group of viruses of the family *Flaviviridae* grouped in the genus *Pestivirus* together with closely related classical swine fever virus (CFSV) and border disease virus (BDV) (Donis, 1995). The virus mainly infects cattle but infections among other animals such as swine, sheep, goat and wild animals are also reported (Liess & Moennig, 1990; Nettleton & Entrican, 1995; Deregt *et al.*, 2005; Vilcek & Nettleton, 2006). CFSV causes a contagious haemorrhagic disease of pigs of worldwide importance and BDV infects sheep causing principally reproductive disease (Vilcek & Nettleton, 2006).

The virus genome is a single stranded positive sense RNA of approximately 12.5 kb. It has a single open reading frame (ORF) that is flanked by a non-coding region (NCR) at the 5' and 3' ends. The ORF is translated into a polyprotein of about 4000 amino acids, which is co- and post-translationally processed by cellular and viral proteases. The mature viral proteins are arranged as follows: NH<sub>2</sub>-N<sup>pro</sup>, C, E<sup>ms</sup>, E1, E2, p7, NS2-3, NS4A, NS4B, NS5A and NS5B-COOH (Meyers & Thiel, 1996; Ridpath, 2005). The 5' NCR, N<sup>pro</sup>, E<sup>ms</sup>, E2 and NS2/3 genome regions have been used to identify pestiviruses, by means of reverse transcription-polymerase chain reaction (RT-PCR) complemented by sequencing for phylogenetic analysis. In particular, the 5' NCR and N<sup>pro</sup> regions show a high degree of conservation among pestiviruses, making them suitable for detection of a broad range of viruses and for strain discrimination studies (Hofmann, Brechtbuhl & Stauber, 1994; Becher *et al.*, 1997; 1999). The E2 protein is a highly genetically and antigenically variable glycoprotein and possesses neutralising epitopes and thus is used to study the molecular and serological diversity of BVDV (Bolin *et al.*, 1988; Corapi, Donis & Dubovi, 1990; Ridpath, Bolin & Dubovi, 1994; Becher *et al.*, 2003). The protein E<sup>ms</sup> remains intracellular and is secreted from infected cells and can be found free in the serum of primary and persistently infected (PI) animals, which gives it the potential to be used as

diagnostic antigen (Brownlie, Thompson & Curwen, 2000). The NS2-3 are conserved and strongly recognized by monoclonal or polyclonal antibodies (Donis, Corapi & Dubovi, 1991), and these proteins are therefore used in antigen detection tests (Sandvik, 2005).

## 1. Genotypes

There are two genetically distinct types of BVDV, BVDV-1 and BVDV-2, which may be differentiated from each other and from other pestiviruses by monoclonal antibodies (MAb) directed against the E2 protein, or by genetic analysis of different regions of the genome (Pellerin *et al.*, 1994; Paton *et al.*, 1995b; Tijssen *et al.*, 1996; Ridpath, 2003;). Recently, an atypical isolate 'HoBi', which was found in a batch of pooled foetal calf serum, has been suggested to be of a third genotype of BVDV (Schirrneier *et al.*, 2004).

Whereas BVDV-1 is the dominant genotype found worldwide, BVDV-2 appears to prevail mainly in the US and Canada. In Europe, Asia and South America, BVDV-2 has been reported sporadically ( Beer, Wolf & Kaaden, 2002; Flores *et al.*, 2002; Park *et al.*, 2004; Cranwell, Jones & Wakeley, 2005; Barros *et al.*, 2006; Pizarro-Lucero *et al.*, 2006). BVDV-2 infections have been associated with outbreaks of a severe acute infection that cause a haemorrhagic syndrome (Rebhun *et al.*, 1989; Carman *et al.*, 1998). However, subclinical infections are common with both genotypes. By molecular studies, BVDV-1 and BVDV-2 are further subdivided into at least 11 subtypes for BVDV-1 and 2 subtypes for BVDV-2 ( Vilcek *et al.*, 2001; Flores, *et al.*, 2002;). This heterogeneity seen among field isolates is a characteristic of RNA viruses, and is probably the result of high mutation rate during viral replication due to the error prone nature of viral RNA polymerase (Bolin & Grooms, 2004). Despite this, BVDV strains isolated at different points in time, from different animals within a herd show a high degree of homology, suggesting that BVDV is genetically stable after being introduced into the herd. This has led to the concept of herd-specific strain of BVDV, and is thought to be related to the presence of immunotolerant, persistently infected (PI) animals within the herd ( Paton *et al.*, 1995a; Hamers *et al.*, 1998; Vilcek *et al.*, 1999).

## 2. Biotypes

BVDV exists in two biotypes, the cytopathic (cp) and the non-cytopathic (ncp) and the classification is based on the ability of the virus to produce cytopathology or not in cultured cells. Cytopathic viruses may arise from ncp viruses after genetic alteration (i.e. insertion, duplication or rearrangements) within the region encoding the NS2/3 protein. The molecular changes result in expression of a NS3 protein ( Brownlie, 1990; Meyers & Thiel, 1996; Baroth *et al.*, 2000; Kümmerer *et al.*, 2000;Becher *et al.*, 2002). Cytopathic biotypes have only been isolated in connection with outbreaks of mucosal disease (MD). The ncp biotype is commonly found in nature and causes persistent infection in animals. For establishment of a persistent infection ncp BVDV escape the cellular defence mechanisms by turning off common responses to virus infection such as apoptosis and/or suppress induction of interferon and production of the Mx protein

(Bendfeldt, Grummer & Greiser-Wilke, 2003; Peterhans, Jungi & Schweizer, 2003; Schweizer *et al.*, 2006).

### *Transmission of BVDV*

Persistently infected animals shed large quantities of BVDV throughout their lives and are the key transmitters of the virus. The main transmission route in infected herds is direct contact with a PI animal (Bolin, 1990; Duffell & Harkness, 1985; Fray, Paton & Alenius, 2000; Lindberg & Alenius, 1999; Niskanen *et al.*, 2000; Tråvén *et al.*, 1991). Indirect transmission usually occurs when virus is well-preserved within the vehicle, e.g. in frozen semen from PI and acutely infected bulls, contaminated embryos or contaminated injection fluids. The potential for such a spread, even over the continents, is high (Schlafer *et al.*, 1990; Kirkland *et al.*, 1991; Falcone, Tollis & Conti, 1999; Barkema *et al.*, 2001; Givens *et al.*, 2003; Niskanen & Lindberg, 2003; Schirrmeyer, *et al.*, 2004; Stringfellow *et al.*, 2005). Indirect transmission by other means such as common calving pens, insects and examination glove, has only been shown experimentally, and only where the initial contact was PI (Tarry, Bernal & Edwards, 1991; Gunn, 1993; Lang-Ree *et al.*, 1994; Mars, Bruschke & van Oirschot, 1999; Niskanen & Lindberg, 2003; Lindberg *et al.*, 2004).

Acutely infected animals do not seem to play any substantial role in the transmission of BVDV (Niskanen, *et al.*, 2000; Niskanen, Lindberg & Tråvén, 2002). If the infected animals are not in the early stage of pregnancy, there is little chance of the virus persisting and those animals seem to be dead-ends of the infection.

### *Clinical manifestation of BVDV*

BVDV causes a wide spectrum of clinical manifestations ranging from subclinical disease to the apparently always fatal form known as mucosal disease (MD). The clinical response to the infection depends on multiple interactive factors including the biotype and/or genotype of the virus, and the immune and pregnancy status of the host.

#### 1. Infection in non-immune non-pregnant animal

Acute BVDV infections in non-immune cattle with BVDV are usually subclinical. However, the consequence of such infections is an immunosuppression (Chase, Elmowalid & Yousif, 2004), which predisposes the animal to infections with other pathogens (Potgieter *et al.*, 1984b; Baker, 1995; Potgieter, 1997; Graham *et al.*, 1998; de Verdier Klingenberg, Vagsholm & Alenius, 1999). The decrease in milk yield, high bulk milk somatic cell count and increased risk of clinical mastitis in dairy cows are associated with BVDV infections and are also believed to be caused, at least in part, by this immunosuppressive effect (Niskanen *et al.*, 1995; Lindberg & Emanuelson, 1997; Waage, 2000; Beaudeau *et al.*, 2004; 2005).

## 2. Congenital infection

In the first trimester of pregnancy, BVDV infection may result in foetal death, followed by abortion or mummification (Bielefeldt-Ohmann, 1995). Essentially, all foetuses surviving this period are born PI, with specific immunotolerance to the persisting virus (Brock & Chase, 2000). The final stages of organogenesis of the nervous system and eye occur during the second trimester and are susceptible to a disruption caused by viral replication. Resultant congenital defects include lens opacity, blindness, retinal dysplasia, cataracts, cerebellar hypoplasia and hydrocephalus. A stillborn calf is another sequel to a congenital infection in the second trimester. After 125 days of pregnancy the foetal immune system has been developed and can produce neutralising antibodies and eliminate the virus. At birth immunocompetent calves are virus-negative and antibody-positive (Moennig & Liess, 1995 ; Graham, 2001).

## 3. Persistent infection

Only transplacental infections by ncp biotypes during the first trimester cause the birth of PI calves (Charleston *et al.*, 2001). After delivery, the PI calves have a specific immunotolerance to their persisting BVDV and do not exhibit any detectable antibody or T cell response to the virus (Collen *et al.*, 2000). The clinical signs vary from the apparently normal health to unthriftiness and most of these calves die or are slaughtered due to disease. Between 1 to 2% of cattle are PI with BVDV in most countries with an intensive beef and dairy production (Houe, 2005). The total losses from BVDV infections in such endemic infected countries due to e.g. death of calves, abortion and subclinical disease have been estimated to be in the range of 10 – 40 million \$ per million calving (Houe, 2003). Therefore infections with BVDV must be regarded as a serious animal welfare problem in many countries all over the world. Some PI calves can survive until maturity and if they are retained for breeding, their offspring is always PI but often fails to survive. PI bulls can produce semen of an acceptable quality, but may be associated with infertility (Moennig & Liess, 1995). However, Voges *et al.* (1998) report a case of a bull that was strongly sero-positive and non-viraemic but persistently shed the virus in the semen. This condition is thought to arise when bulls become infected at puberty, i.e. during the formation of the immunological 'blood-testes' barrier, thus allowing the virus to replicate within the testes and evade immune surveillance (Voges, *et al.*, 1998; Fray, Paton & Alenius, 2000).

## 4. Mucosal disease

Only PI cattle are at risk of developing MD (Bolin, 1995b; Brownlie, Clarke & Howard, 1984; Roeder & Drew, 1984). Mucosal disease is characterised by a high death rate (100%) usually occurring within 1-2 weeks after the onset of clinical signs. Post-mortem examinations reveal erosions in the mucosa at various sites along the gastrointestinal tract (Baker, 1987). The cp biotypes are always found together with the homologous ncp biotype that persists in the affected animal. The cp strain can be of exogenous origin (i.e. be introduced through experiments or vaccines) or arise *de novo* by a genetic change in the resident ncp virus (Ridpath & Bolin, 1995; Meyers & Thiel, 1996; Fritzemeier *et al.*, 1997).

### *Laboratory diagnosis of BVDV infections*

The diagnostic assays, are in general, aimed to detect virus specific antibodies as an indication of exposure to BVDV, and/or aim at detection of infectious virus or viral components as an indication of current infection (review in: Sandvik, 2005)

#### 1. Detection of immune response against BVDV

Primary infection in immunocompetent animals results in seroconversion after approximately three weeks (Howard, 1990). The antibody levels rise slowly, until a plateau is reached 10–12 weeks after infection and BVDV will probably be detected for the rest of the life of host (Brownlie *et al.*, 1987; Fredriksen *et al.*, 1999). Different serological tests have been adopted for BVDV serology and the widely used sensitive tests are the virus neutralisation test and the enzyme linked immunosorbent assays (ELISAs).

##### 1.1 Virus neutralisation test

The virus neutralisation (VN) test is the gold standard test for the detection of BVDV, CSFV and BDV antibodies because of its high sensitivity and specificity. The antibodies detected by the VN are predominantly against E2, which may result in different titres depending on which virus strain is used in the assay (Jones *et al.*, 2001; Couvreur *et al.*, 2002). No single strain is ideal for all circumstances. For detection of antibodies to BVDV, a strain that detects the highest proportion of serological reactions in the local cattle population should be selected. Many laboratories use highly laboratory-adapted cp strains, such as Oregon C24V and NADL, even though immune labelling techniques are now available that allow detection of neutralisation of ncp strains. The output is based on the highest serum dilution that inhibits replication of a quantified amount of the challenge virus. However, the test is laborious and takes 5–6 days to perform, requires experienced staff and well equipped laboratories, reagents and cell culture free from contamination of pestivirus. It is therefore mostly used as a reference test for back-up and calibration purposes (Sandvik, 1999; 2005). It is also a crucial test to be able to differentiate, by use of cross neutralisation, the serological response in animals infected with other pestiviruses.

##### 1.2 The enzyme-link immunosorbent assays (ELISA)

For testing of large number of samples, ELISA has many advantages over the VN. The test is independent of cell cultures and challenge viruses, gives a test result within a few hours, is relatively inexpensive both to establish and run, and is suitable for automation. There are two format of ELISA, the indirect and the blocking or competitive type, which are used for detection of antibodies in milk, blood plasma and serum samples (Howard, Clarke & Brownlie, 1985; Katz & Hanson, 1987; Niskanen *et al.*, 1991; Paton *et al.*, 1991; Schrijver & Kramps, 1998). Indirect ELISA kits often use the whole BVDV as antigen for measuring antibody response against the full spectrum of immunogenic BVDV proteins. Blocking ELISA kits may use individual viral proteins and MAb, which may be a limitation (or advantage) since e.g. some ELISA tests have not been able to detect any antibodies in animals vaccinated with e.g. inactivated vaccines (Graham *et al.*, 2003).

The determination of the level of antibodies in pooled samples as bulk tank milk and 'spot test' can give a useful indication of the BVDV status of a herd (Niskanen, *et al.*, 1991; Niskanen, 1993; Houe *et al.*, 1995; Paton *et al.*, 1998; Lindberg & Alenius, 1999; Pritchard, 2001; Valle *et al.*, 2005). In addition, the immune response in pregnant cows, measured with an indirect ELISA can also be used to predict the BVDV status of their foetuses since in late pregnancy the dam with a PI foetus often has a markedly higher antibody response than does a cow carrying an uninfected foetus (Alenius *et al.*, 1992; Brownlie *et al.*, 1998; Lindberg *et al.*, 2001).

## 2. Detection of BVDV

In principle, there are three different methods of detecting the virus or viral components: virus isolation in cell culture, detection of viral antigens and detection of viral nucleic acid. In primary infected animals, BVD virus and antigen can be detected in blood from the first couple of days to around two weeks after infection whereas viral RNA could be detected even longer (Sandvik, Fredriksen & Løken, 1997; Bruschke *et al.*, 1998; Ellis *et al.*, 1998). In PI animals, BVDV antigen can be detected in sera and tissue samples during the whole life after maternal antibody has disappeared.

### 2.1 Virus isolation (VI) in cell culture

The virus may be isolated in a number of bovine monolayer cell cultures particularly in primary or secondary bovine cultured cells e.g. kidney, lung, testis or turbinate cells (Edwards, 1990; Saliki *et al.*, 1997). Buffy coat cells or serum and semen are suitable for isolation of the virus from live animals. Maternal antibodies may however interfere with the VI in young calves. For detection of cp strains, the cells are inoculated with samples and checked for a cytopathic effect. For ncp strains, an immune labelling i.e. immunoperoxidase technique (IPX), described by Meyling (1984), in the final step is necessary. Virus isolation is an essential back-up and reference test for other indirect methods for identification of PI animals, and should be available as a confirmatory test for laboratories that use other tests to detect BVDV indirectly.

### 2.2 Detection of viral antigens

The *antigen-capture ELISA* is based on either MAb specific to one or more viral antigens for both capture and detection of antigen, or a combination of polyclonal and MAbs (Fenton *et al.*, 1991; Mignon *et al.*, 1992; Shannon, Mackintosh & Kirkland, 1993; Shannon *et al.*, 1991). It is essential that these MAbs recognise all antigenic variants of BVDV circulating in the bovine population to be tested. In PI animals, BVDV antigens can be readily be detected in tissue sections, such as skin biopsies, by *immunohistochemical staining* (Thür, Zlinszky & Ehrensperger, 1996; Spagnuolo-Weaver *et al.*, 1997; Brodersen, 2004; Loneragan *et al.*, 2005). Although more labour intensive than antigen ELISAs, this method can be applied to detect viral antigen in young PI calves, which otherwise could have tested negatively by VI or antigen ELISAs due to inhibition by maternal antibodies (Zimmer *et al.*, 2004).

### 2.3 Reverse transcription –polymerase chain reaction

To detect viral nucleic acid of BVDV, reverse transcription-polymerase chain reaction (RT-PCR) has been adapted and used widely. Detection of RNA by RT-PCR comprises of four different steps: extraction of RNA, reverse transcription to complementary DNA, primer-directed amplification and lastly detection of amplified products. In the past, each step was run separately which was time-consuming and increased risk of contamination of samples, especially when many samples are processed together. The closed one-tube TaqMan RT-PCRs combine the last three steps in a single tube, and by eliminating the need for gel electrophoresis, the risk of carry-over contamination with the previously amplified products and false positives is greatly reduced. The RT-PCR technique has been used to detect BVDV in various samples such as foetal fluids, tissues, serum, semen, cell cultures and whole blood (Belák & Ballagi-Pordány, 1991; Da Silva *et al.*, 1995; Letellier *et al.*, 1999; Lindberg *et al.*, 2002; Stokstad *et al.*, 2003). The *nested RT-PCR* is another modified techniques of RT-PCR which re-amplifies the first PCR product and therefore increases test sensitivity (Gruber *et al.*, 1994). The development of the *real-time PCR* provides the capability to quantify the virus in pooled blood serum, and to genomic typing by using specific sets of primers and probes (Letellier & Kerkhofs, 2003; Baxi *et al.*, 2006; Willoughby *et al.*, 2006).

### *Control of BVDV*

Different disease control schemes of BVDV are used in different countries. They can be divided into non-vaccination and vaccination schemes.

#### 1. Non-vaccination schemes

Eradication programmes are in progress in a number of European countries including Sweden, Norway, Denmark, Finland and Austria with successful performance (Bitsch, Hansen & Ronshølt, 2000; Hult & Lindberg, 2005; Rikula *et al.*, 2005; Rossmannith, Janacek & Wilhelm, 2005; Valle, *et al.*, 2005). Based on the Scandinavian experience, effective protocols have been described for herd screening followed by identification and removal of PI animals and/or certification of a BVDV-free status (Lindberg & Alenius, 1999). In accordance with the programme protocol, herds may be certified free from infection based on low levels of or undetectable antibody in repeatedly taken bulk milk samples or absence of serum antibodies in small numbers of home-bred young animals 6-18 months old, taken at least twice with a more than 6-month interval (Alenius, Lindberg & Larsson, 1997). High levels of bulk milk antibody and seropositivity in a spot test, by contrast, indicate an active BVDV infection. This is often in the form of one or more PI animals present in the herd. Identification and removal of PI animals in a safe and effective way from a herd requires individual testing of all animals in the herd (Larsson *et al.*, 1994). At all stages of the procedure the farmers receive information and advice, based on the current knowledge of the BVDV status of the herd (Lindberg & Alenius, 1999). The principal risk factors for introduction or reintroduction of an infection are purchase of PI animals or dams carrying PI foetus or contact with PI cattle from an infected herd (Alenius, *et al.*, 1992; Alenius, Lindberg & Larsson, 1997; Bitsch, Hansen & Ronshølt, 2000).



Management procedures need also to be reviewed in order to ensure that an adequate biosecurity is provided and that any reintroduction is detected and dealt with rapidly (Graham, 2001; Valle, *et al.*, 2005).

## 2. Vaccination schemes

The primary goal of vaccination is to induce a sufficient level of immunity sufficient to prevent transplacental infection of foetuses and subsequent birth of PI animals. The combination of vaccines and systematic identification, and removal of PI animals can be used to eradicate BVDV from herds. The vaccination ensures that the herd protection due to the natural infection wanes; it is replaced by a vaccine-induced immunity (Moennig *et al.*, 2005). However, there are difficulties in implementing such vaccination programmes (Graham, 2001). Notably, in countries that have made extensive use of live BVDV vaccines, such as US, Canada, Belgium, Germany and Japan, a high presence of BVDV type 2 infections has been reported (Pellerin, *et al.*, 1994; Bolin, 1995a; ; Wolfmeyer *et al.*, 1997; Letellier, *et al.*, 1999; Ridpath *et al.*, 2000; Nagai *et al.*, 2001; Tajima *et al.*, 2001). This is in contrast to results obtained from studies performed in countries that have not used live BVDV vaccines, such as Sweden, UK, New Zealand and Australia. Consequently, there appears to be an association between the use of live BVDV vaccines and the presence of type 2 BVDV strains in the cattle populations (Lindberg and Alenius, 1999). Furthermore, live cp vaccines can induce MD in PI animals by recombination between vaccine virus and the ncp-persistent virus (Fritzemeier *et al.*, 1995; Ridpath & Bolin, 1995; Becher, Orlich & Thiel, 2001). Moreover, modified live vaccine may cause reproductive disease, immunosuppression and ovarian lesions (Bolin, 1995a; Grooms, Brock & Ward, 1998). Inactivated BVDV vaccines are safe to use in pregnant animals but their efficacy and potency, e.g. to prevent congenital infection seems limited (van Oirschot, Bruschke & van Rijn, 1999; Graham *et al.*, 2004). In Germany, a BVDV control programme based on testing and, as an option a two-step vaccination (with inactivated and live vaccines) is underway (Moennig, *et al.*, 2005; Moennig, Houe & Lindberg, 2005).

## Bovine herpesvirus type 1

### *The virus*

Bovine herpesvirus type 1 (BHV-1) is a member of the family *Herpesviridae*, subfamily *Alphaherpesvirinae* and is an important pathogen of cattle (Ackermann & Engels, 2006). By restriction enzyme analysis, two subtypes of BHV-1 have been described. BHV-1 subtype 1 represents strains that cause respiratory disease, infectious bovine rhinotracheitis (IBR), whereas subtype 2 includes strains that cause genital diseases, e.g. infectious pustular vulvovaginitis (IPV) and infectious balanoposthitis (IBP) (Engels, Steck & Wyler, 1981; Radostits *et al.*, 2000). Metzler *et al.* (1985) used DNA restriction enzyme analysis to further classify subtype 2 into 2a and 2b. Subtypes 1 and 2a have been isolated from aborted foetuses, whereas the less virulent subtype 2b has not been reported to be associated with abortion (Edwards, Newman & White, 1991; Miller, Whetstone &

Van der Maaten, 1991). By conventional serological assays there is however only one antigenic serotype of BHV-1. Infection with BHV-1 has also been reported to occur in wild ruminants and swine (Varady, Tuboly & Derbyshire, 1994 ; Radostits, *et al.*, 2000).

#### *Transmission of BHV-1*

The virus is most commonly transmitted by close contact between infected and susceptible animals by means of trade (Nylin, Madsen & Ronshølt, 1998; Hage *et al.*, 2003). Indirect transmission may occur via contaminated equipment and semen ( van Oirschot *et al.*, 1993; van Oirschot, 1995; Hage, *et al.*, 2003).

#### *Clinical manifestation of BHV-1*

Infectious bovine rhinotracheitis (IBR) usually occurs as a subclinical respiratory infection but also can be a cause of a severe disease. Morbidity and mortality may reach 100% and 10%, respectively, particularly if complication occurs (Radostits, *et al.*, 2000). Unilateral or bilateral conjunctivitis, often with profuse lacrimation, is a common clinical sign of the infection although bronchitis and bronchiolitis have occasionally been observed. In most cases pulmonary pathology has not been found unless secondary bacterial bronchopneumonia occurs (Radostits, *et al.*, 2000). Secondary bacterial infections with, for example, *Pasteurella* spp., can give rise to more severe clinical signs due to deeper airways being affected. Abortions may occur in the course of IBR during the third trimester of pregnancy (Murphy *et al.*, 1999). Where natural mating is practiced, genital infection can lead to pustular vulvovaginitis or balanoposthitis. These are characterized by mild to severe necrotic lesions in the vaginal or preputial mucosae. After artificial insemination with infected semen, endometritis can arise (Kendrick & McEntress, 1987). Uncomplicated cases of respiratory or genital disease caused by BHV-1 are usually last 5-10 days.

As a member of the herpesvirus family, BHV-1 can cause latency. The sciatic and trigeminal ganglia and the tonsils are the sites of latency after genital and respiratory disease, respectively (Ackermann, Peterhans & Wyler, 1982; Ackermann & Wyler, 1984; Winkler, Doster & Jones, 2000). Latency allows the virus to persist, so that the introduction of a latent infected carrier into a non-infected herd is the principal way for the virus to spread. The persisting BHV-1 can be reactivated and re-excreted by several stimuli, including transport, parturition, and treatment with glucocorticoids (Thiry *et al.*, 1987). When BHV-1 is reactivated, the virus establishes a new life cycle of replication and can be transmitted to susceptible animals (Bitsch, 1973; van Oirschot, *et al.*, 1993).

#### *Laboratory diagnosis of BHV-1 infections*

The BHV-1 infection can be identified by detection of a specific antibody response and detection of the virus or viral antigens.

## 1. Detection of immune response against BHV-1

Specific antibodies to BHV-1 can be first detected at 7 to 10 days post-infection. After the acute phase and during latency, BHV-1-infected cattle are mainly detected by the presence of specific antibodies to BHV-1. Virus neutralisation tests and various ELISAs are usually used for detecting antibodies against BHV-1 in serum and ELISA tests appear to gradually be replacing VN tests. Several types of BHV-1 ELISA tests are commercially available and most of them are also suitable for detecting antibodies in milk. The test on bulk milk, used with an appropriate cut-off value, has been reported to estimate the prevalence of BHV-1 in a herd (Pritchard, 2001). However, data from individual or pooled serum from non-milking groups will more precisely provide the herd's status. In addition, some ELISAs can be used in conjunction with marker vaccines to detect infected cattle in a vaccinated population (van Oirschot *et al.*, 1997; Wellenberg *et al.*, 1998).

## 2. Detection of BHV-1

The virus can be isolated from nasal swabs taken during the acute phase of the infection, semen from infected bulls and from various organs collected at post-mortem (Radostits, *et al.*, 2000). However, the isolation of BHV-1 from an animal does not mean that the virus is the cause of the disease outbreak because it may be a latent virus that has been reactivated under stress conditions. A confirmatory laboratory diagnosis must be made on a group of animals and must be accompanied by either seroconversion from negative to positive or a fourfold or higher rise of antibody titres to BHV-1.

### 2.1 Virus isolation in cell culture

Primary and secondary bovine kidney, lung, testis, turbinate, trachea or foetal lung cells and established cell lines are all suitable for virus isolation. The selected cells are inoculated with the samples and are observed daily for cytopathic effect. An alternative method of virus identification is the direct demonstration of BHV-1 antigen in infected cells by an immunofluorescence or conjugated immunoperoxidase assay based on monoclonal antibodies (Kaashoek *et al.*, 1994).

### 2.2 Detection of viral antigens

The fluorescence antibody test is used during the acute stage of the disease and is appropriate for detecting presence of the virus in nasal, ocular and genital swabs. Scraping of mucosal lesions or white plaques from the nasal mucosa should give a positive and rapid result in most acute cases. However, the sensitivity of this procedure is lower than that of virus isolation (Edwards, Chasey & White, 1983).

### 2.3 Polymerase chain reaction (PCR)

The PCR has been developed to detect BHV-1 DNA in infected semen samples (van Engelenburg *et al.*, 1993; 1995; Vilcek *et al.*, 1994; Xia, Yason & Kibenge, 1995; Grom *et al.*, 2006). The PCR assay is more sensitive and independent of the quality of the sample than either virus isolation or the fluorescent antibody tests, and it is also faster than virus isolation (Vilcek, *et al.*, 1994; Moore, Gunn & Walls, 2000; Grom, *et al.*, 2006).

### *Control of BHV-1*

BHV-1 is included in the list B disease of the OIE and it is therefore a target for international legislation and control. At the national/regional/herd level, the virus is either controlled systematically by 'test-and-cull' or 'test-and-removal' with or without vaccination schemes.

'Test and cull' without vaccination has been used with a high degree of success in countries such as Denmark, Finland, Austria, Sweden and Switzerland (Ackermann *et al.*, 1989;1990; Nylin, Madsen & Ronshølt, 1998; Kofer, Wagner & Deutz, 1999; Vonk Noordegraaf *et al.*, 2000; Paisley, Tharaldsen & Jarp, 2001). For systematic schemes, biosecurity is the main important factor for the successful eradication of BHV-1. Biosecurity of BHV-1 is based on hygienic measures. Ideally, a 4-week quarantine period is imposed for introduced cattle to a free herd. Only seronegative animals after the quarantine are can be admitted into the herd. Once an animal is infected it has been regarded as a lifelong potential shedder of BHV-1 and therefore can be culled or removed from the herd. To minimise the risk of introducing the virus into BHV-1-free herds or AI centres, the recognition and removal of animals that are latent carriers is important in control programmes and in international trade activities (Kramps *et al.*, 1996). Control with testing and vaccination has been applied in e.g. Germany, Belgium, Hungary, a province in Italy and the Netherlands (Tanyi & Varga, 1992; Nardelli *et al.*, 1999;Limbourg *et al.*, 2002;Trapp, König & Beer, 2003; Vonk Noordegraaf *et al.*, 2004). However, not a single country that has included vaccination has yet succeeded to eradicate BHV-1 (Ackermann & Engels, 2006).

Although clinical disease induced by BHV-1 can be reduced by vaccination, latent infections cannot be prevented (Kaashoek, *et al.*, 1994). Another disadvantage of conventional vaccines is their interference with routine serological diagnostic tests. Marker vaccines reduce this problem but the serology must be based on compatible diagnostic tools (Kaashoek, *et al.*, 1994;Babiuk, van Drunen Littel-van den Hurk & Tikoo, 1996; van Oirschot, *et al.*, 1997;de Wit *et al.*, 1998; Wellenberg, *et al.*, 1998).

In many countries no systematic action at all are taken on the herd or regional level to reduce the BVDV and BHV-1 infections, the prophylaxis is based on 'herd's decision' of using multivalent BVDV and BHV-1 vaccines. Furthermore in some countries, no action at all has been taken in the past to try to prevent these infections.

## Aims

The overall aim of this study was to investigate the epidemiological pattern of BVDV and BHV-1 infections in dairy herds in Thailand. More specifically, the following aims were set:

- To establish the prevalence and relationship between BVDV and BHV-1 infections in dairy herds in northern and north-eastern Thailand (I).
- To study the incidence of BVDV and BHV-1 infections in a long-term study of dairy herds in north-eastern Thailand, both on an individual and herd level (II).
- To evaluate the usefulness of different serological and virological methods for BVDV investigations in dairy cattle herds (I-V).
- To characterize BVDV strains in Sweden and Thailand and to investigate whether such a molecular approach may help to trace routes of transmission (IV, V).
- To discuss recommendations for a future control of BVDV and BHV-1 infections in Thailand based on the performed studies.

## Materials and methods

Detailed description of material and methods are given in each paper.

### Study design

#### *Paper I & II*

In Paper I, 220 dairy herds were randomly selected during 2000-2001 from nine MCs: seven located in the north-eastern provinces; Khon Kaen, Udon Thani and Sakon Nakorn (MC 1-7), and two in a northern province; Chiang Mai (MC 8-9). These herds represented approximately 20% of the dairy herds in the nine MCs (range: 10-36%). From this set of herds, a subset of 11 herds located in the Khon Kaen province (MC 1 and 2) was also selected. This selection was based on accessibility. Blood samples were collected from all cattle in these dairy herds and analyzed to detect the presence or absence of current infections. Bulk tank milk from the herds was collected at the day of blood collection. In Paper II the sampling of these 11 herds was repeated in September 2003 and October-November 2004. During 2002-2004, 186 herds were BTM collected four times from MC 1-7 in the north-eastern provinces. Among this group, 67 herds had been investigated in 2000. During the period, vaccination against BVDV and BHV-1 still was not used in the study areas.

#### *Paper III*

A commercial E<sup>ms</sup> capture ELISA (Herd Check BVDV Ag/Serum plus, IDEXX Laboratories, INC.) was evaluated in comparison with the VI test for detection of BVDV antigen in cattle sera. Two hundred-twenty cattle sera from routine submissions in the Swedish BVDV eradication programme were selected. According to the results from the IPX used within the routine diagnostics (Meyling, 1984) 90 of the sera were considered virus positive and 130 virus negative.

To study the influence of passive immunity on the performance of the ELISA, 23 serum samples were used from 9 PI calves born after a previously described experimental infection of pregnant heifers (Lindberg, *et al.*, 2002). The samples were collected between day 0 (i.e. immediately after birth and before intake of colostrums) and day 11 post partum. Of the nine calves, five were given colostrums free from BVDV antibodies, and four were given colostrums from their respective antibody positive dams.

The influence of heat inactivation was studied on a subset of 20 sera (10 virus positive and 10 virus negative) out of the 220 samples previously selected from the routine diagnostics. Each sample was divided in two parts, and one was heat inactivated at 56°C for 90 minutes before further analysis. Heat inactivated and non-heated sera were then tested in parallel with the E<sup>ms</sup> ELISA.

#### *Paper IV*

In Paper IV, sera from individuals identified as virus-positive within the Swedish BVDV eradication programme between October 2002 and June 2004 were collected and stored at -20°C. During the 18 months, 329 individuals from 112 herds were tested virus-positive and isolates from all positive samples were stored in the BVDV bank and used later for phylogenetic analysis.

### **Diagnostic tests**

#### *Indirect ELISA for BVDV*

A commercial indirect ELISA kit (SVANOVA Biotech AB, Uppsala, Sweden) was used to detect antibodies to BVDV in bulk milk and serum in Paper I and II, according to the instructions of the manufacturer. The corrected optical density (COD) level was calculated by subtracting the optical density (OD) for the control antigen from sample OD;  $(OD_{\text{sample}} - OD_{\text{control}}) = \text{COD}$ . In Paper I the Swedish system of classification and interpretation of the results of the BVDV antibody ELISA on bulk milk was used whereby herds are allocated into four different classes based on COD levels (Niskanen, 1993; Lindberg & Alenius, 1999). The individual results were interpreted according to Juntti *et al.* (1987), i.e. sera with CODs  $\geq 0.20$  were considered positive.

In Paper II, percent positivity (PP) was used instead of the CODs to minimize the effect of plate variability between analyses. The PP was calculated as followed:  $PP = \text{COD}_{\text{sample}} / \text{mean COD}_{\text{positive control}} \times 100$ . Individual serum results were interpreted according to the manufacturer; i.e. sera with  $PP \geq 14$  were considered positive. The cut-off for PP-values in BTM samples that were able to classify herds with the highest accuracy was selected based on its sensitivity and specificity. The 95% confidence intervals (CI) of the sensitivity and specificity estimates, as well as positive and negative predictive values, were also calculated. All herd BTM samples were subsequently classified as positive for BVDV if the PP-value was higher than the selected cut-off ( $PP \geq 2$ ). Additionally, BTM samples positive for BVDV was further differentiated as medium and high antibody herds; when the PP-value ranged between 13 – 30 and greater than or equal to 30, respectively, which correspond to BVDV class 2 and 3 in the Swedish BVDV eradication programme (Niskanen, 1993; Jalali *et al.*, 2005).

#### *Indirect ELISA for BHV-1*

A commercial indirect ELISA kit (SVANOVA Biotech AB, Uppsala, Sweden) was used in Paper I and II to detect antibodies to BHV-1 in bulk milk and in serum, according to the instructions of the manufacturer. The corrected optical density (COD) level was calculated before interpretation of the results by subtracting the optical density (OD) for the control antigen from sample OD ( $OD_{\text{sample}} - OD_{\text{control}} = \text{COD}$ ). In Paper I, the results of the BHV-1 antibody ELISA on bulk milk were interpreted according to the Swedish IBR control programme, i.e. samples with CODs  $\geq 0.05$  were considered positive. In Paper II the cut-off value ( $PP \geq 3$ ) for classification of positivity / negativity of bulk milk samples was

established in the same way as described for BVDV. The individual sera with CODs  $\geq 0.20$  were considered positive for antibodies to BHV-1.

### *IN*

The neutralisation capacity of the serum samples was tested to the two accepted pestivirus species; BVDV-1 (NADL), BVDV-2 (CVL-178003/GB-1987) and to the recently described atypical BVDV strain D32/00\_ 'HoBi'. In Paper V, a border disease virus (BDV) strain (strain 137/4) was included in the test protocol. A volume of 50  $\mu$ l of serial two-fold dilution, from 1:8 of heat inactivated sera were mixed in two to four wells in the microtitre plates with 50  $\mu$ l with approximately 100 TCID<sub>50</sub> of respective pestivirus strain and incubated 1 hour at 37°C in a humidified CO<sub>2</sub> incubator. Approximately 15,000 bovine turbinate cells were added per well. After 3 days of incubation at 37°C in a humidified CO<sub>2</sub> incubator, the plates were fixed and dried before they were stained with immunoperoxidase technique as described by Meyling (1984).

### *E<sup>ms</sup> capture ELISA*

A commercial E<sup>ms</sup> capture ELISA (Herd Check BVDV Ag/Serum plus, IDEXX Laboratories INC.) was evaluated in Paper III, and used to detect BVDV antigen in cattle sera in Paper I-II and V, according to the manufacturers' instructions. The relative sensitivity and specificity of the E<sup>ms</sup> ELISA in relation to IPX was calculated and the degree of agreement between the IPX and the E<sup>ms</sup> ELISA was measured by determining the kappa ratio. To estimate the effect of heat inactivation on the performance of the ELISA, the repeatability of the test before and after heat inactivation of the samples used the concordance correlation coefficient (Lin, 1989) and Bland-Altman plot (Bland & Altman, 1986).

### *RT-PCR and genotyping*

In Paper IV, one isolate from each of the 112 infected herds was selected for amplification and sequencing. BVDV RNA was extracted from the supernatants of virus-infected cells using a fully automated one-step protocol. The target region, 5'NCR was amplified as described by Elvander *et al.* (1998). A 237-nucleotide fragment of the 5'NCR, corresponding to position 135-371 of the BVDV-1 strain SD-1 (Deng & Brock, 1992), was used for phylogenetic analysis. Additional sequences representative of each known genetic subgroup of BVDV were obtained from the GenBank and included in the alignments. Phylogenetic trees were constructed using neighbour-joining. The robustness of the method was evaluated by bootstrap analysis.

In Paper V BVDV RNA was extracted directly from the antigen positive serum. All other procedures were performed as described above. The phylogenetic analyses was done in the 183 nucleotides fragment of 5' NCR, corresponding to position 160-341 of the BVDV-1 strain NADL (Colett *et al.*, 1988).



## Results and discussion

A detailed description and discussion of the results are given in each paper.

### **Prevalence of BVDV and BHV-1 infections in dairy herds in northern and north-eastern Thailand** (Paper I)

The results from BTM testing demonstrated a moderate level of exposure to BVDV and BHV-1 in the 220 studied dairy herds from the northern and north-eastern Thailand, with prevalences of antibody-positive herds of 73 and 67%, respectively. One of the two MC from the northern province (MC 9) had low prevalences of BVDV (14%) and BHV-1 (2%) compared with 80% and 96% of the neighbouring MC 8. Demography and management practices were not studied but one distinguishing factor was that most herds in MC 9 were established about 10 years earlier than the herds in MC 9. At the animal level the low within-herd prevalence of BVDV and BHV-1 in the 11 herds (24 and 5%, respectively), particularly among the young stock (15 and 0%, respectively), indicated a low prevalence of active BVDV infections and a low rate of reactivation of latent BHV-1.

### **Evidence of a self-clearance process of BVDV infection** (Paper II)

The long-term study of the 186 dairy herds showed that the prevalence of BVDV positive herds decreased from 91% in 2002 to 72% in 2004 ( $\chi^2 = 24.36$ ,  $p \leq 0.001$ ). The prevalence of BVDV positive herds of the 67 herds decreased from 94% in 2000 to 75% in 2004 ( $\chi^2 = 9.54$ ,  $p \leq 0.01$ ). Similarly, the proportion of class 3 BVDV herd of the 186 herds significantly decreased from 20% in 2002 to 8% in 2004 ( $\chi^2 = 12.9$ ,  $p \leq 0.001$ ) and for the subset of 67 herds from 22% in 2000 to 4% in 2004 ( $\chi^2 = 6.08$ ,  $p \leq 0.025$ ).

The clearance process was also shown at the animal level in the 11 herds. In 2001, all 11 herds had antibody-positive animals. A total of 83 antibody-positive animals were found with a mean seroprevalence of 24% (range 6-55%). During the study period the prevalence decreased in 9 out of 10 (90%) of the herds where all animals were sampled. The incidence rate was low among cattle in 10 out of 11 herds. One herd, however, had a high seroconversion rate to BVDV and a viraemic calf was identified by a commercial E<sup>ms</sup> capture ELISA. Genetic characterization (Paper V) showed that the calf was infected with a virus closely related to atypical pestivirus strain HoBi that was recently detected by Schirrneier *et al.* (2004), in a batch of foetal calf serum originating from Brazil. The comparative VN test strongly suggests that the seroconversions in these herd and two other herds were caused by the 'HoBi'-like virus strain. Furthermore, a retrospective study indicated the virus has recently been introduced to the region.

### **Evidence of a self-clearance process of BHV-1 infection (Paper II)**

The prevalence of BHV-1 positive herds, based on 186 herds of MC 1-7, decreased significantly from 61% in 2002 to 48% in 2004 ( $\chi^2 = 6.24$ ,  $p \leq 0.025$ ). Over a longer time period the prevalence in the 67 herds decreased from 84% in 2000 to 57% in 2004 ( $\chi^2 = 11.55$ ,  $p < 0.001$ ). The likelihood of herds free of BHV-1 infection in 2002 being antibody positive in 2004 was 0.29 (21/73). On the other hand, the likelihood of seropositive herds becoming free of BHV-1 infection during the same period was 0.40 (45/113). There were 43 herds free of BHV-1 in both 2002 and 2003, and 37 herds of these remained free to the end of this study. Six herds became positive in 2004 but only one herd had a high PP value (PP=86) whereas the other five remained low (PP ranged from 3 to 15, mean 7.2).

The incidence rate of BHV-1 in the 11 herds during 2001- 2004 was low. Only seven animals in three herds seroconverted during the study period and three of these seroconversions occurred in a herd totally free from antibody-positive animals. The four other seroconversions occurred in two herds with BHV-1 infected animals present and that reactivated virus caused these infections cannot be excluded. However, based on this study and also other studies (van Nieuwstadt & Verhoeff, 1983; Hage, *et al.*, 2003), the risks of reactivation of BHV-1 infected animals probably have been greatly overestimated in the past (Pritchard, 1992 ; Kampa *et al.*, 2004). One explanation might be that different strains of BHV-1 have a higher tendency for reactivation than others.

Herd characteristics such as low number of purchased animals, small herd size and low incidence of ongoing BVDV infections may also be beneficial for the self-clearance process of BHV-1. Furthermore treatments of cows with corticosteroids in Thai dairy herds are not a common practice.

Since it has been calculated that the uncertainty of the yearly reactivation rate of latently BHV-1 infected animals greatly affects the eradication costs of BHV-1 from a cattle population ( Vonk Noordegraaf *et al.*, 2002; Ackermann & Engels, 2006) this is an important area of research that needs to be studied in carefully controlled field studies or under well controlled experimental conditions.

### **Relationship between BVDV and BHV infection (Paper I and II)**

There was a significant association between being antibody-positive to BVDV and being BHV-1-positive, at the herd level both in the initial study (Paper I) and the long-term study (Paper II). In the long-term study, the relationship between BHV-1 antibody status and BVDV antibody status in the 186 BTM samples was highly significant at all four samplings. At an animal level, in the 11 closely monitored herds, there were 31 cattle that had antibodies to BHV-1 during 2001-2004. Seventeen of these were also seropositive to BVDV. One animal became infected with both viruses in 2004. The strong significant association between being antibody positive to the viruses, both at the herd and animal level, suggests that similar factors might be of importance for the risk of introduction of both these infections to dairy herds. Such a strong association on a herd level has also been reported from a study performed in the UK (Paton, *et al.*, 1998).

One obvious risk factor, and probably the most important, is the introduction of dually infected cattle to a herd, while another risk factor is artificial inseminations. It was observed that all seven seroconversions to BHV-1 in the 11 herds occurred in animals older than 2 years (range 2-10 years) which had all been inseminated. For BVDV 14 cows that had all been inseminated seroconverted to BVDV. These seroconversions may have been caused by BHV-1 and/or BVDV contaminated sperm or by indirect transmission to the animals by the person performing the inseminations. However, the specific route could not be traced by the herd's records because the animals were inseminated several times by different practitioners using different batches of semen, but this factor could not be ruled out.

### **Use of indirect ELISA to estimate herd's BVDV and BHV-1 status (Paper I and II)**

In BVDV and BHV-1 eradication schemes, antibody testing of pooled milk and pooled serum sample has been used intensively in the Scandinavian countries as herd screening tests. In a non-vaccinated population a high antibody level in BTM using an indirect ELISA indicates a probability of the herd having been exposed to BVDV in the recent past. By contrast, an absence of antibodies indicates that the herd is free from the infection (Graham, 2001; Niskanen, 1993; Paton, *et al.*, 1998).

In Paper I and II a commercial indirect ELISA (SVANOVA Biotech AB) was used to determine BVDV and BHV-1 infection status of the herds by BTM which yielded highly valuable information. The study of BTM in Paper I was based on a COD classification system that is used in the Swedish BVDV and BHV-1 eradication programmes (Lindberg & Alenius, 1999).

In Paper II, PP was used instead of COD values to reduce the effect of inter-plate variability. The test characteristics for the indirect BVDV ELISA at a cut-off for PP of 2 showed a sensitivity of 100% (CI 88-100%) as 28 BTM samples from herds with seropositive lactating cows all tested positive. The specificity of the test was 80% (CI 28-100%) as one false positive (PP = 2.7) was found among the five negative lactating groups. The evaluated cut-off at 2 PP is the same as the cut-off value used in the Swedish BVDV-eradication scheme for BVDV class 0 herds (Jalali, *et al.*, 2005; Niskanen, *et al.*, 1991). The corresponding cut-off value for the indirect BHV-1 ELISA (SVANOVA Biotech AB) was 3 PP which is close to the COD value at 0.05 that is used in the Swedish monitoring programme for freedom of BHV-1 infection.

### **Comparative virus neutralisation test for BVDV (Paper II and V)**

Individual susceptible cattle respond with higher neutralising titre to the genotype of BVDV with which they have been infected and with comparative lower titres to other genotypes (Jones, *et al.*, 2001; Couvreur, *et al.*, 2002; Schirrmeier, *et al.*, 2004). The results of the comparative neutralisation test in Paper II & V indicated the need to include the 'HoBi' strain in a reference panel. If the HoBi had not been

included in the neutralisation tests, it would have been hard to explain the serological pattern against BVDV type 1 and type 2 found in the individual animals in three of the herds that seroconverted with more than fourfold higher titres against HoBi compared to BVDV type 1 and 2.

The low titre to the HoBi, but high against BVDV-1, in all analysed sera collected in 2001 implies that the atypical virus was recently introduced during 2002-2003. Thus the test can be used to investigate retrospectively which genotype of BVDV an animal has most probably been infected with.

### **Antigen ELISA (Paper III)**

In a regional or national control programme for BVDV it is important that the tests used are robust, have a high sensitivity and specificity and have been thoroughly evaluated for use on routine samples submitted to the laboratory. In Paper III the results from 220 field serum samples in this study showed the ability of the commercial E<sup>ms</sup>ELISA (IDEXX Laboratories, INC.) to detect BVDV antigen in cattle serum. The test has 100% relative sensitivity and specificity when compared to the virus isolation. Heat inactivation did not disrupt the ELISA performance. An atypical pestivirus (Th/04\_KhonKaen) in heat inactivated calf serum was detected by the test.

### **RT-PCR and genotyping (Paper IV and V)**

Molecular epidemiology uses molecular data as the basis for investigations to trace sources of infection. In Paper IV, one isolate from each of the 112 infected herds was selected for amplification and sequencing. Phylogenetic analysis showed that the BVDV strains that circulate in Sweden belong to genotype 1: subtype 1a (n=7), 1b (n=28) or 1d (n=77), according to the nomenclature used by Vilcek *et al.* (2001), confirming previous results. Alignment and comparison of the 5' NCR fragment revealed a total of 67 different sequences, and among these 48 were unique for a single herd. Nineteen sets of identical sequences originated from isolates from between 2 and 14 different herds. The percentage of identity between pairs of sequences varied between 95-100% within subtypes and between 85-91% between subtypes, corresponding to differences of between 0-13 nucleotides (nts) and 21-35 nts, respectively. In three cases, suspected routes of transmission were supported by the phylogenetic analysis. Since this paper was published, more isolates have been sequenced and the method helped to both strengthen suspected routes of transmission and also rule out suspicions.

The antigen positive calf serum from Thailand was analysed in Paper V. The analysis demonstrated that the detected virus (Th/04\_KhonKaen) was closely related to D32/00\_'HoBi'. Pairwise evolutionary distance within the studied fragment between Th/04\_KhonKaen and D32/00\_HoBi was estimated at 0.08. The range of pairwise distances between Th/04\_KhonKaen and other species within the genus *Pestivirus*, including the pestivirus of giraffe, were 0.28-0.43.

### **Suggestion for BVDV & BHV-1 control in Thailand**

This study provides evidence of a pronounced self-clearance process of both BVDV and BHV-1 infections. Based on experiences from Scandinavian countries and on the results in this study, this process can continue as long as introductions of the infections to non-infected herds are prevented. There seem to be no need to use vaccines for the control of the infections. The importance of biosecurity regarding e.g. animal introductions, insemination practices and treatments must be stressed. Repeated analysis of bulk tank milk has shown its effectiveness in many control programmes. Results from this low-cost test can indicate the status of the herds i.e. having an active infection (high antibody level) or a clearance of the virus (negative antibody status). The data must be provided to the farmers as a help when purchasing new animals i.e. only choose animals from BVDV-free and BHV-1-free herds. The traded animals should also have sufficient information i.e. origin of the animal, vaccination and breeding history. Based on the herd data, local authorities must be made aware of the transmission routes of the viruses from infected herds to the free-herds. All bull stations in the country that produce sperm should aim to be BVDV and BHV-1 free in the future. Other biological products made within the country for use in the animal populations should be shown to be free from virus contaminations. Imported animals into the country and biological products should also be shown to be free from both BVDV and BHV-1. Moreover it would be interesting to perform studies of the present pestivirus status in other animal populations e.g. beef cattle, swine and buffalo.

## Concluding remarks

Based on the studies presented in this thesis the following conclusions can be drawn:

- The prevalence of BVDV and BHV-1 infections in north-eastern Thailand was significantly decreased, resulting from a self-clearance process. The self-clearance will most probably continue as long as there is awareness, among farmers, veterinarians and government authorities of herd biosecurity issues. There seem to be no reasons to use vaccines for the control of these infections in Thailand.
- The available diagnostic techniques, comparative neutralisation tests, virus isolation, antibody and antigen ELISA tests and molecular techniques can be used as powerful tools for pestivirus investigations and in the control of such infections.
- Further studies must be performed to elucidate the extent of reactivation of BHV-1 under different field conditions and how the atypical HoBi-like BVDV strain entered to the cattle population in Thailand.

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